## REMARKS

### Amendments

Claims 1-33 have been canceled, claims 34 and 41 have been amended, and claims 43-48 have been added. Upon entry of the amendment, claims 34-37 and 41-48 will be pending. Support for the added claims can be found in the specification, for example, on page 8, lines 15-18; Figure 2B; and in the claims as originally filed.

The foregoing amendments are made solely to expedite prosecution of the application and are not intended to limit the scope of the invention. Further, the amendments to the claims are made without prejudice to the pending or now canceled claims or to any subject matter pursued in a related application. The Applicant reserves the right to prosecute any canceled subject matter at a later time or in a later filed divisional, continuation, or continuation-in-part application.

### Rejections

***Rejections under 35 U.S.C. § 101/112 1st paragraph***

The Examiner has rejected claims 34-36, 41 and 42 because the claimed invention is allegedly not supported by either a specific or substantial asserted utility or a well-established utility.

Applicant respectfully traverses the rejection. Amended claim 1 is drawn to a transgenic mouse whose genome comprises a null endogenous anaphylatoxin C3a receptor allele, where the allele comprises SEQ ID NO:1; the null allele comprises exogenous DNA; and the exogenous DNA comprises a gene encoding a visible marker. According to 35 U.S.C. § 101, "[w]hoever invents . . . any new and useful . . . composition of matter may obtain a patent therefore. . . . "

Under the Patent Office's Utility Requirement Guidelines:

> If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible.
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> If the applicant has asserted that the claimed invention is useful for any particular practical purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.

(emphasis added)(MPEP § 2107, II (A)(3); II (B)(1)). Thus, according to Patent Office guidelines, a rejection for lack of utility may not be imposed where an invention has a well-established utility or is useful for any particular practical purpose. The present invention satisfies either standard.

The present invention has a well-established utility since a person of ordinary skill in the art "would immediately appreciate why" knockout mice are useful. As a general principle, any knockout mouse has the inherent and well-established utility of defining the function and role of the disrupted target gene, regardless of whether the inventor has described any specific phenotypes, characterizations or properties of the knockout mouse. The sequencing of the human genome has produced countless genes whose function has yet to be determined. According to the National Institute of Health, knockout mice represent a critical tool in studying gene function:

> Over the past century, the mouse has developed into the premier mammalian model system for genetic research. Scientists from a wide range of biomedical fields have gravitated to the mouse because of its close genetic and physiological similarities to humans, as well as the ease with which its genome can be manipulated and analyzed.
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> In recent decades, researchers have utilized an array of innovative genetic technologies to produce custom-made mouse models for a wide array of specific diseases, as well as to study the function of targeted genes. One of the most important advances has been the ability to create transgenic mice, in which a new gene is inserted into the animal's germline. Even more powerful approaches, dependent on homologous recombination, have permitted the development of tools to "knock out" genes, which involves replacing existing genes with altered versions; or to "knock in" genes, which involves altering a mouse gene in its natural location. To preserve these extremely valuable strains of mice and to assist in the propagation of strains with poor reproduction, researchers have taken advantage of state-of-the-art reproductive technologies, including cryopreservation of embryos, in vitro fertilization and ovary transplantation.

(http://www.genome.gov/pfv.cfm?pageid=10005834) (emphasis added). Thus, the knockout mouse has been accepted by the NIH as the premier model for determining gene function, a utility that is specific, substantial and credible.

Knockout mice are so well accepted as tools for determining gene function that the director of the NIH Chemical Genomics Center of the National Human Genome Research Institute (among others, including Capecchi, Bradley, Joyner, Nagy and Skarnes) has proposed creating knockout mice for all mouse genes:

> Now that the human and mouse genome sequences are known, attention has turned to elucidating gene function and identifying gene products that might have therapeutic value. The laboratory mouse (Mus musculus) has had a prominent role in the study of human disease mechanisms throughout the rich, 100-year history of classical mouse genetics, exemplified by the lessons learned from naturally occurring mutants such as agouti, reeler and obese. The large-scale production and analysis of induced genetic mutations in worms, flies, zebrafish and mice have greatly accelerated the understanding of gene function in these organisms. Among the model organisms, the mouse offers particular advantages for the study of human biology and disease: (i) the mouse is a mammal, and its development, body plan, physiology, behavior and diseases have much in common with those of humans; (ii) almost all (99%) mouse genes have homologs in humans; and (iii) the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem (ES) cells, allowing genes to be altered efficiently and precisely.
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> A coordinated project to systematically knock out all mouse genes is likely to be of enormous benefit to the research community, given the demonstrated power of knockout mice to elucidate gene function, the frequency of unpredicted phenotypes in knockout mice, the potential economies of scale in an organized and carefully planned project, and the high cost and lack of availability of knockout mice being made in current efforts.

(Austin et al., Nature Genetics (2004) 36(9):921-24, 921)(emphasis added)(copy attached).

With respect to amended claims drawn to transgenic mice having a null allele, the following comments from Austin are relevant:

> **Null-reporter alleles should be created**
> The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (e.g., P-galactosidase or green fluorescent protein) allows a rapid assessment of which cell types normally

support the expression of that gene.

(p. 922)(emphasis added).

According to the Molecular Biology of the Cell (Albert, 4th ed., Garland Science (2002)), one of the leading textbooks in the field of molecular biology:

> Extensive collaborative efforts are underway to generate comprehensive libraries of mutation in several model organisms including . . . the mouse. The ultimate goal in each case is to produce a collection of mutant strains in which every gene in the organism has either been systematically deleted, or altered such that it can be conditionally disrupted. Collections of this type will provide an invaluable tool for investigating gene function on a genomic scale.

(p. 543)(emphasis added).

According to Genes VII (Lewin, Oxford University Press (2000)), another well respected textbook in the field of genetics:

> The converse of the introduction of new genes is the ability to disrupt specific endogenous genes. Additional DNA can be introduced within a gene to prevent its expression and to generate a null allele. Breeding from an animal with a null allele can generate a homozygous "knockout", which has no active copy of the gene. This is a powerful method to investigate directly the importance and function of the gene.

(p. 508)(emphasis added).

Research tools such as knockout mice are clearly patentable, as noted by the Patent Office:

> Some confusion can result when one attempts to label certain types of inventions as not being capable of having a specific and substantial utility based on the setting in which the invention is to be used. One example is inventions to be used in a research or laboratory setting. Many research tools such as gas chromatographs, screening assays, and nucleotide sequencing techniques have a clear, specific and unquestionable utility (e.g., they are useful in analyzing compounds). An assessment that focuses on whether an invention is useful only in a research setting thus does not address whether the invention is in fact "useful" in a patent sense. Instead, Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Labels such as "research tool," "intermediate" or "for research purposes" are not helpful in determining if an applicant has identified a specific and substantial utility for the invention.

(MPEP § 2107.01, I). As with gas chromatographs, screening assays and nucleotide sequencing techniques, knockout mice have a clear, specific and unquestionable utility (e.g., they are useful in analyzing gene function).

In addition, commercial use and acceptance is an important indication that the utility of an invention has been recognized by one of skill in the art ("A patent system must be related to the world of commerce rather than to the realm of philosophy." *Brenner v Manson*, 383 U.S. 519, 148 U.S.P.Q. 689, 696 (1966)). Commercial use of the knockout mice produced by Assignee Deltagen has been clearly established. At least four (4) large pharmaceutical companies have ordered the presently claimed transgenic mouse. This commercial acceptance more than satisfies the practical utility requirement of section 101 as it cannot be reasonably argued that a claimed invention which is actually being commercially used has no use (see, for example, Phillips Petroleum Co. v. U.S. Steel Corp., 673 F. Supp. 1278, 6 U.S.P.Q.2d 1065, 1104 (D. Del. 1987), *aff'd*, 865 F.2d 1247, 9 U.S.P.Q.2d 1461 (Fed. Cir. 1980)("lack of practical utility cannot co-exist with infringement and commercial success); (Lipscomb's Walker on Patents, §5:17, p. 562 (1984)("Utility may be evidenced by sales and commercial demand.")

Applicant submits that since one of ordinary skill in the art would immediately recognize the utility of a knockout mouse in studying gene function, a utility which is specific, substantial and credible, the invention has a well-established utility, thus satisfying the utility requirement of section 101. On this basis alone, withdrawal of the rejection with respect to the present invention is warranted, and respectfully requested.

In addition, the claimed invention is useful for a particular purpose. The claimed mice exhibit reduced thymus weight, reduced thymus size, reduced thymus to body weight ratio, increased susceptibility to seizure and/or a stimulus processing deficit. One of skill in the art would recognize that these mice are useful for studying the association of the anaphylatoxin C3a receptor gene with any one of these phenotypes.

The Examiner argues that there is no evidence that the anaphylatoxin C3a receptor gene or the cited phenotypes are associated with any disease. The Examiner's arguments are similar to arguments made by the Patent Office with respect to pharmaceutical compounds the utility of which were based on murine model data, arguments which were dismissed by the Federal Circuit in *In re Brana* (34 U.S.P.Q.2d 1436)(Fed. Cir. 1995). The case involved compounds that were disclosed to be effective as anti-tumor agents and had demonstrated activity against murine

lymphocytic leukemias implanted in mice. The court ruled that the PTO had improperly rejected, for lack of utility, claims for pharmaceutical compounds used in cancer treatment in humans, since neither the nature of invention nor evidence proferred by the PTO would cause one of ordinary skill in art to reasonably doubt the asserted utility.

The first basis for the Board's holding of lack of utility (the Board adopted the examiner's reasoning without any additional independent analysis) was that the specification failed to describe any specific disease against which the claimed compounds were useful, and therefore, absent undue experimentation, one of ordinary skill in the art was precluded from using the invention. (*In re Brana* at 1439-40). The Federal Circuit reasoned that the leukemia cell lines were originally derived from lymphocytic leukemias in mice and therefore represented actual specific lymphocytic tumors. The court concluded that the mouse tumor models represented a specific disease against which the claimed compounds were alleged to be effective. (*In re Brana* at 1440).

The Board's second basis was that even if the specification did allege a specific use, the applicants failed to prove that the claimed compounds were useful.

The Federal Circuit responded: "[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of Section 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." (*Brana* at 1441, *citing In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971)). From this it followed that the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. (*Id.*)

The court held that the Patent Office had not met its burden. The references cited by the Board did not question the usefulness of any compound as an antitumor agent or provide any other evidence to cause one of skill in the art to question the asserted utility of applicants' compounds. Rather, the references merely discussed the therapeutic predictive value of *in vivo* murine tests -- relevant only if the applicants were required to prove the ultimate value in

humans of their asserted utility. The court did not find that the nature of the invention alone would cause one of skill in the art to reasonably doubt the asserted usefulness. The purpose of treating cancer with chemical compounds did not suggest an inherently unbelievable undertaking or involve implausible scientific principles. (*Id.*)

The Court concluded that one skilled in the art would be without basis to reasonably doubt the asserted utility on its face. The PTO had not satisfied its initial burden. Accordingly, the applicants should not have been required to substantiate their presumptively correct disclosure to avoid a rejection under the first paragraph of Section 112. (*Id.*)

As in *Brana*, Applicant has asserted that the claimed invention is useful for a particular practical purpose, an assertion that would be considered credible by a person of ordinary skill in the art. As discussed above, the claimed mice have demonstrated specific phenotypes. The acceptance among those of skill in the art of knockout mice demonstrating such properties is clearly demonstrated.

Definitive proof that the phenotypes observed in the null mouse would be the same as those observed in humans is not a prerequisite to satisfying the utility requirement. It is enough that knockout mice are recognized in the art as models for determining gene function. As noted by Austin et al.:

> Among the model organisms, the mouse offers particular advantages for the study of human biology and disease: (i) the mouse is a mammal, and its development, body plan, physiology, behavior and diseases have much in common with those of humans; (ii) almost all (99%) mouse genes have homologs in humans; and (iii) the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem (ES) cells, allowing genes to be altered efficiently and precisely.

(p. 921)(emphasis added).

In addition, as pointed out by Doetschman, one clearly skilled in the art, (*Laboratory Animal Science* 49:137-143, 137 (1999)(copy attached), the phenotypes observed in mice correlate well to gene function:

> The conclusions will be that the knockout phenotypes do, in fact, provide accurate information concerning gene function . . .

(emphasis added).

Moreover, the tests used by Applicant to determine the asserted phenotypes are well recognized by those skilled in the art. In *Brana*, the claimed compound had demonstrated

activity against a murine tumor implanted in a mouse. Yet, the Federal Circuit found that utility had been demonstrated. Here, the invention relates to a disruption in a murine gene in a mouse. Like the tumor mouse model, the knockout mouse with a specific gene disrupted is a widely accepted model, the utility of which would be readily accepted in the art. It is submitted that one skilled in the art would be without basis to be reasonably doubt Applicant's asserted utility, and therefore the Examiner has not satisfied the initial burden.

In addition, the claimed transgenic mice are useful for studying gene expression. The mice within the scope of claim 43 contain a visible marker such as lacZ. Their use in studying gene expression is clearly recognized by those skilled in the art:

> **Null-reporter alleles should be created**
> The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (e.g., P-galactosidase or green fluorescent protein) allows a rapid assessment of which cell types normally support the expression of that gene.

(Austin et al., Nature Genetics (2004) 36(9):921-24, 922)(emphasis added)(copy attached). Applicant reminds Examiner that a claimed invention need only satisfy one of its stated objectives to satisfy the utility and enablement requirements.

In summary, Applicant submits that the claimed transgenic mouse, regardless of any disclosed phenotypes, has inherent and well-established utility in the study of the function of the gene, and thus satisfies the utility requirement of section 101. Moreover, Applicant believes that the transgenic mice are useful for studying anaphylatoxin C3a receptor gene function with respect to the cited phenotypes as well as studying gene expression; and are therefore useful for a specific practical purpose that would be readily understood by and considered credible by one of ordinary skill in the art.

In light of the arguments set forth above, Applicant does not believe that the Examiner has properly established a *prima facie* showing that establishes that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the Applicant would be specific and substantial. (*In re Brana*; MPEP § 2107).

Withdrawal of the rejections is respectfully requested.

***Rejections under 35 U.S.C. § 112, 1st paragraph***

Claims 34-37, 41 and 42 have been rejected for lack of enablement, as the claimed invention allegedly lacks utility. As set forth above, it the Applicant's position the claimed invention satisfies the utility requirement and therefore one skilled in the art would clearly know how to use the invention. Withdrawal of the rejections is respectfully requested.

The claims have also been rejected for encompassing a heterozygous mouse which does not exhibit a phenotype. The Examiner argues that one skilled in the art would not know how to use such a mouse.

Applicant disagrees. The claimed mice contain a visible marker and are therefore useful for gene expression analysis (see Example 2). The mice are also useful for breeding homozygous mice and are preferred by Deltagen's customers. Applicant submits that a claim to a novel composition of matter need not recite a property or phenotype in order to be patentable. In addition, a claimed invention need only satisfy one stated goal or objective to satisfy the utility and enablement requirements. The claimed invention has several, including determining gene function, studying specific phenotypes, studying gene expression profiles and breeding homozygous mice.

Withdrawal of the rejection is respectfully requested.

In view of the above amendments and remarks, Applicant respectfully requests a Notice of Allowance. If the Examiner believes a telephone conference would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. 13-2725.

Respectfully submitted,

11-24-04

Date

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# The Knockout Mouse Project

**Mouse knockout technology provides a powerful means of elucidating gene function *in vivo*, and a publicly available genome-wide collection of mouse knockouts would be significantly enabling for biomedical discovery. To date, published knockouts exist for only about 10% of mouse genes. Furthermore, many of these are limited in utility because they have not been made or phenotyped in standardized ways, and many are not freely available to researchers. It is time to harness new technologies and efficiencies of production to mount a high-throughput international effort to produce and phenotype knockouts for all mouse genes, and place these resources into the public domain.**

Now that the human and mouse genome sequences are known[1–3], attention has turned to elucidating gene function and identifying gene products that might have therapeutic value. The laboratory mouse (*Mus musculus*) has had a prominent role in the study of human disease mechanisms throughout the rich, 100-year history of classical mouse genetics, exemplified by the lessons learned from naturally occurring mutants such as agouti[4], reeler[5] and obese[6]. The large-scale production and analysis of induced genetic mutations in worms, flies, zebrafish and mice have greatly accelerated the understanding of gene function in these organisms. Among the model organisms, the mouse offers particular advantages for the study of human biology and disease: (i) the mouse is a mammal, and its development, body plan, physiology, behavior and diseases have much in common with those of humans; (ii) almost all (99%) mouse genes have homologs in humans; and (iii) the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem (ES) cells, allowing genes to be altered efficiently and precisely.

The ability to disrupt, or knock out, a specific gene in ES cells and mice was developed in the late 1980s (ref. 7), and the use of knockout mice has led to many insights into human biology and disease[8–11]. Current technology also permits insertion of 'reporter' genes into the knocked-out gene, which can then be used to determine the temporal and spatial expression pattern of the knocked-out gene in mouse tissues. Such marking of cells by a reporter gene facilitates the identification of new cell types according to their gene expression patterns and allows further characterization of marked tissues and single cells.

Appreciation of the power of mouse genetics to inform the study of mammalian physiology and disease, coupled with the advent of the mouse genome sequence and the ease of producing mutated alleles, has catalyzed public and private sector initiatives to produce mouse mutants on a large scale, with the goal of eventually knocking out a substantial portion of the mouse genome[12,13]. Large-scale, publicly funded gene-trap programs have been initiated in several countries, with the International Gene Trap Consortium coordinating certain efforts and resources[14–17].

Despite these efforts, the total number of knockout mice described in the literature is relatively modest, corresponding to only ~10% of the ~25,000 mouse genes. The curated Mouse Knockout & Mutation Database lists 2,669 unique genes (C. Rathbone, personal communication), the curated Mouse Genome Database lists 2,847 unique genes, and an analysis at Lexicon Genetics identified 2,492 unique genes (B.Z., unpublished data). Most of these knockouts are not readily available to scientists who may want to use them in their research: for example, only 415 unique genes are represented as targeted mutations in the Jackson Laboratory's Induced Mutant Resource database (S. Rockwood, personal communication).

The converging interests of multiple members of the genomics community led to a meeting to discuss the advisability and feasibility of a dedicated project to produce knockout alleles for all mouse genes and place them into the public domain. The meeting took place from 30 September to 1 October 2003 at the Banbury Conference Center at Cold Spring Harbor Laboratory. The attendees of the meeting are the authors of this paper.

### Is a systematic project warranted?

A coordinated project to systematically knock out all mouse genes is likely to be of enormous benefit to the research community, given the demonstrated power of knockout mice to elucidate gene function, the frequency of unpredicted phenotypes in knockout mice, the potential economies of scale in an organized and carefully planned project, and the high cost and lack of availability of knockout mice being made in current efforts. Moreover, implementing such a systematic and comprehensive plan will greatly accelerate the translation of genome sequences into biological insights. Knockout ES cells and mice currently available from the public and private sectors should be incorporated into the genome-wide initiative as much as possible, although some may be need to be produced again if they were made with suboptimal methods (e.g., not including a marker) or if their use is restricted by intellectual property or other constraints. The advantages of such a systematic and coordinated effort include efficient production with reduced costs; uniform use of knockout methods, allowing for more comparability between knockout mice; and ready access to mice, their derivatives and data to all researchers without encumbrance. Solutions to the logistical, organizational and informatics issues associated with producing, characterizing and distributing such a large number of

*The Comprehensive Knockout Mouse Project Consortium**

**Authors and their affiliations are listed at the end of the paper.*

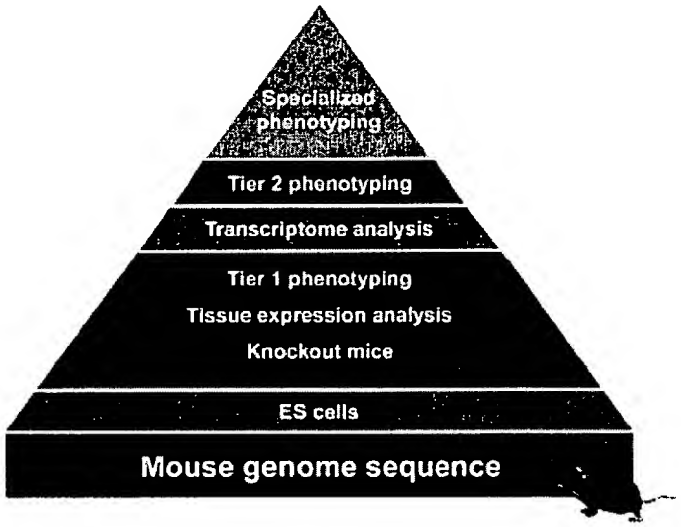


**Figure 1** Structure of resource production in the proposed KOMP. Using the mouse genome sequence as a foundation, knockout alleles in ES cells will be produced for all genes. A subset of ES cell knockouts will be used each year to produce knockout mice, determine the expression pattern of the targeted gene in a variety of tissues and carry out screening-level (Tier 1) phenotyping. In a subset of mouse lines, transcriptome analysis and more detailed system-specific (Tier 2) phenotyping will be done. Finally, specialized phenotyping will be done on a smaller number of mouse lines with particularly interesting phenotypes. All stages will occur within the purview of the KOMP except for the specialized phenotyping, which will occur in individual laboratories with particular expertise.

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mice will draw from the experience of related projects in the private sector and in academia, which have made or phenotyped hundreds of knockout mice using a variety of techniques. Lessons learned from these projects include the need for redundancy at each step to mitigate pipeline bottlenecks and the need for robust informatics systems to track the production, analysis, maintenance and distribution of thousands of targeting constructs, ES cells and mice.

### Null-reporter alleles should be created

The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (*e.g.*, β-galactosidase or green fluorescent protein) allows a rapid assessment of which cell types normally support the expression of that gene. Therefore, we propose to produce a null-reporter allele for each gene. Making each mutation conditional in nature by adding *cis*-elements (*e.g.*, *lox*P or FRT sites) would be desirable, but we do not advocate this as part of the mutagenesis strategy unless the technological limitations currently associated with generating conditional targeted mutations on a large scale and in a cost-effective manner can be overcome.

### A combination of methods should be used

Various methods can be used to create mutated alleles, including gene targeting, gene trapping and RNA interference. Advantages of conventional gene targeting include flexibility in design of alleles, lack of limitation to integration hot spots, reliability for producing complete loss-of-function alleles, ability to produce reporter knock-ins and conditional alleles, and ability to target splice variants and alternative promoters. BAC-based targeting has the potential advantages of higher recombination efficiencies and flexibility for producing complex mutated alleles[18]. Gene trapping is rapid, is cost-effective and produces a large variety of insertional mutations throughout the genome but can be somewhat less flexible[17,19–21]. There is uncertainty regarding the percentage of gene traps that produce a true null allele and the fraction of the genome that can ultimately be covered by gene-trap mutations. Trapping is not entirely random but shows preference for larger transcription units and genes more highly expressed in ES cells. In recent studies, gene trapping was estimated to potentially produce null alleles for 50–60% of all genes, perhaps more if a variety of gene-trap vectors with different insertion characteristics is used[17,21]. RNA interference offers enormous promise for analysis of gene function in mice[22] but is not yet sufficiently developed for large-scale production of gene modifications capable of reliably producing true null alleles. Both gene-targeting and gene-trapping methods are suitable for producing large numbers of knockout alleles, and, given their complementary advantages, a combination of these methods should be used to produce the genome-wide collection of null-reporter alleles most efficiently.

### What should the deliverables be?

A genome-wide knockout mouse project could deliver to the research community a trove of valuable reagents and data, including targeting and trapping constructs and vectors, mutant ES cell lines, live mice, frozen sperm, frozen embryos, phenotypic data at a variety of levels and detail, and a database with data visualization and mining tools. At a minimum, we believe that a comprehensive genome-wide resource of mutant ES cell lines from an inbred strain, each with a different gene knocked out, should be produced and made available to the community. Choosing an inbred line (129/SvEvTac or C57BL/6J), and evaluating the alternative of using $F_1$ ES cells and tetraploid aggregation to provide potential time savings, merits additional scientific review and discussion[23,24]. ES cells should be converted into mice at a rate consistent with project funding and the ability of the worldwide scientific community to analyze them. Although the value and cost-effectiveness of systematically characterizing the mice is a matter of debate, a limited set of broad and cost-effective screens, probably including assessment of developmental lethality, physical examination, basic blood tests, and histochemical analysis of reporter gene expression, would be useful. More detailed phenotyping, based on findings from the initial screen or existing knowledge of the gene's function, could be done at specialized centers. All ES cell clones and mice (as frozen embryos or sperm) should be available to any researcher at minimal cost, and all mouse phenotyping and reporter expression data should be deposited into a public database.

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In determining how to implement the project, utility to the research community should be the standard for judging value. Each step after ES cell generation (e.g., mouse creation, breeding, expression analysis, phenotyping) will make the resource useful to more researchers but will also increase costs and scientific complexity. We therefore advocate a 'pyramid' structure for the project (Fig. 1). At the base of the pyramid is the genome-wide collection of mutant ES cells for every mouse gene. Over time, a subset of these mutant ES cells should be made into mice and characterized with an initial phenotype screen (Tier 1; Fig. 1) and analysis of tissue reporter-gene expression. A subset of these lines should be profiled by microarray analysis, and a subset of these profiled by system-specific (Tier 2) phenotyping, based on the results of the Tier 1 phenotyping, array studies, existing knowledge of the gene's function and the gene's tissue expression pattern. With time, the upper tiers of the pyramid will be filled out, eventually transforming the pyramid into a cube, with information of all types available for all genes.

This project will require the resolution of numerous intellectual property claims involving the production and use of knockout mice. To deal with the existing patents that cover the technologies and processes involved in the production of mutant mice, we suggest that a 'patent pool', such as that used in the semiconductor industry[25], should be generated. Several individuals who represent entities that control patents on mouse knockout technologies are authors on this paper, and they agree with this approach. We also agree that any mutant ES cells or mice produced should be placed immediately in the public domain.

## Mechanisms and costs

**ES cell production.** Automated knockout construct and ES cell production should be carried out in coordinated centers to ensure efficiency and uniformity. We estimate that most known mouse genes could be knocked out in ES cells within 5 years, using a combination of gene-trapping and gene-targeting techniques. Gene trapping can produce a large number of mutated alleles quickly, but its progress should be monitored closely to determine when its yield of new genes diminishes[17] and, therefore, when targeting should be increasingly relied on. As large-scale trapping projects have already defined gene classes that probably cannot be knocked out by trapping (*e.g.*, single-exon GPCRs, genes that are not expressed in ES cells), we propose that targeting begin on those classes immediately. All ES cells should be made available to the research community, because this collection itself would be a valuable resource. Efforts in the public and private sectors have already knocked out many genes in ES cells, and, to the degree that the alleles produced fit the prescribed characteristics (*i.e.*, null alleles with a reporter) and are available, every effort should be made to incorporate these into the planned public resource. Costs for generating this part of the resource were estimated at between $9–11 million/year for five years (these and all subsequent figures are direct costs).

**Mouse production.** The subset of ES cells made into mice each year should be chosen by a peer-review process. Central facilities for high-efficiency mouse production, genotyping, breeding, maintenance and archiving should be funded, to take advantage of efficiencies of scale in mouse creation and distribution. Researchers could apply to produce groups of mice outside the centers, as long as they meet the cost specifications of the program. All mice should be made available immediately to researchers as frozen embryos or sperm, for nominal distribution cost. An initial target of 500 new mouse lines per year would double the current rate at which new genes are knocked out in the public sector; we feel that this rate is within the capacity of the biomedical research community worldwide to absorb and analyze. We estimated the initial cost of this level of mouse production to be $12.5–15 million per year.

**Reporter tissue expression analysis.** Approximately 30 tissues from adult and developmental stages should be sampled to cover the main organ systems. Analysis methods should be customized to the organ system and marker, and a searchable database of the sites of gene expression, and the images showing them, should be produced. Centers to carry out these analyses and data curation should be selected by peer review. We estimated the cost of this component for 500 mouse lines to be $2.5–5 million per year, depending on how much tissue sectioning and cell-level analysis is done.

**Phenotyping.** Tier 1 phenotyping should be a low-cost screen for clear phenotypes and should be done on all mouse lines produced. Tier 1 should include home-cage observation, physical examination, blood hematological and chemistry profiles, and skeletal radiographs. The centers producing the mice should carry out the Tier 1 analyses, at an estimated cost of $2.5 million per year for 500 lines. Selected lines, chosen on the basis of findings from Tier 1 phenotyping, tissue expression patterns, microarray data and the scientific literature, should undergo more detailed and system-focused Tier 2 phenotyping. Tier 2 phenotyping should be done in specialized phenotyping centers, akin to those already in operation for phenotyping of mice produced by ENU mutagenesis. All Tier 1 and Tier 2 phenotyping should be done on a uniform genetic background by dedicated groups of individuals in single locations, to facilitate consistency and cross-comparison of results among different mouse lines. All Tier 1 and Tier 2 phenotyping results should be deposited into a central project database freely accessible to the research community. More detailed and specialized phenotyping could be done by individual researchers in their own laboratories; deposition of this more detailed phenotype data would be encouraged.

**Transcriptome analysis.** Transcriptome profiling of tissues from each knockout line, collected in a uniform way across all mice and tissues and placed into a searchable relational database, would add substantially to the scientific value of the project, though it would also add considerably to its cost. Transcriptome analysis should therefore be done on a subset of mice, chosen by peer review. We estimate that, with the best currently available array technology, an analysis of ten tissues would cost ~$18,000 per line.

## Conclusions

This project, tentatively named the Knockout Mouse Project (KOMP), will be a crucial step in harnessing the power of the genome to drive biomedical discovery. By creating a publicly available resource of knockout mice and phenotypic data, KOMP will knock down barriers for biologists to use mouse genetics in their research. The scientific consensus that we achieved—that a dedicated project should be undertaken to produce mutant mice for all genes and place them into the public domain—is important but is only the beginning. Implementation of these recommendations will require additional input from the greater scientific community, including those responsible for programmatic direction and financial support of biomedical research in the public and private sectors. This ambitious and historic initiative must be carried out as a collaborative effort of the worldwide scientific community, so that all can contribute their skills, and all can benefit. International discussions among scientific and programmatic staffs since the Banbury meeting at Cold Spring Harbor, in both the public and private sectors, have shown that there is great enthusiasm and commitment to this vision. The next step for KOMP will be to move this visionary plan from conceptualization to implementation, with an urgency befitting the benefits it will bring to science and medicine.

URLs. The curated Mouse Knockout & Mutation Database is available at http://research.bmn.com/mkmd/. The curated Mouse Genome Database is available at http://www.informatics.jax.org/. *Patent pools: A solution to the problem of access in biotechnology patents?* is available at http://www.uspto.gov/web/offices/pac/dapp/opla/patentpool.pdf.


1. International Human Genome Sequencing Consortium. *Nature* 409, 860-921 (2001).
2. Venter, J.C. *et al. Science* 291, 1304-1351 (2001).
3. Mouse Genome Sequencing Consortium. *Nature* 420, 520-562 (2002).
4. Buttman, S.J., Michaud, E.J. & Woychik, R.P. *Cell* 71, 1195-1204 (1992).
5. D'Arcangelo, G. *et al. Nature* 374, 719-723 (1995).
6. Zhang, Y. *et al. Nature* 372, 425-432 (1994).
7. Goldstein, J.L. *Nat. Med.* 7, 1079-1080 (2001).
8. D'Orleans-Juste, P., Honore, J.C., Carrier, E. & Labonte, J. *Curr. Opin. Pharmacol.* 3, 181-185 (2003).
9. Horton, W.A. *Lancet* 362, 560-569 (2003).
10. Wallace, D.C. *Am. J. Med. Genet.* 106, 71-93 (2001).
11. Chen, R.Z., Akbarian, S., Tudor, M. & Jaenisch, R. *Nat. Genet.* 27, 327-331 (2001).
12. Zambrowicz, B.P. *et al. Nature* 392, 608-611 (1998).
13. Nadeau, J.H. *et al. Science* 291, 1251-1255 (2001).
14. Wiles, M.V. *et al. Nat. Genet.* 24, 13-14 (2000).
15. Stryke, D. *et al. Nucleic Acids Res.* 31, 278-281 (2003).
16. Hansen, J. *et al. Proc. Natl. Acad. Sci. USA* 100, 9918-9922 (2003).
17. Skarnes, W.C. *et al. Nat. Genet.* 36, 543-544 (2004).
18. Valenzuela, D.M. *et al. Nat. Biotechnol.* 21, 652-659 (2003).
19. Chen, W.V., Delrow, J., Corrin, P.D., Frazier, J.P. & Soriano, P. *Nat. Genet.* 36, 304-312 (2004).
20. Stanford, W.L., Cohn, J.B. & Cordes, S.P. *Nat. Rev. Genet.* 2, 756-768 (2001).
21. Zambrowicz, B.P. *et al. Proc. Natl. Acad. Sci. USA* 100, 14109-14114 (2003).
22. Kunath, T. *et al. Nat. Biotechnol.* 21, 559-561 (2003).
23. Seong, E., Saunders, T.L., Stewart, C.L. & Burmeister, M. *Trends Genet.* 20, 59-62 (2004).
24. Eggan, K. *et al. Nat. Biotechnol.* 20, 455-459 (2002).
25. Clark, J., Piccolo, J., Stanton, B. & Tyson, K. Patent pools: A solution to the problem of access in biotechnology patents? (US Patent and Trademark Office, 2000).



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Special Topic Overview

# Interpretation of Phenotype in Genetically Engineered Mice

Thomas Doetschman

***Background and Purpose:*** **In mice, genetic engineering involves two general approaches—addition of an exogenous gene, resulting in transgenic mice, and use of knockout mice, which have a targeted mutation of an endogenous gene. The advantages of these approaches is that questions can be asked about the function of a particular gene in a living mammalian organism, taking into account interactions among cells, tissues, and organs under normal, disease, injury, and stress situations.**

***Methods:*** **Review of the literature concentrating principally on knockout mice and questions of unexpected phenotypes, lack of phenotype, redundancy, and effect of genetic background on phenotype will be discussed.**

***Conclusion:*** **There is little gene redundancy in mammals; knockout phenotypes exist even if none are immediately apparent; and investigating phenotypes in colonies of mixed genetic background may reveal not only more phenotypes, but also may lead to better understanding of the molecular or cellular mechanism underlying the phenotype and to discovery of modifier gene(s).**

One often hears the comment that genetically engineered mice, especially knockout mice, are not useful because they frequently do not yield the expected phenotype, or they don't seem to have any phenotype. These expectations are often based on years of work, and in some instances, thousands of publications of mostly in vitro studies. Examples of unexpected phenotypes, based largely on experience with transforming growth factor beta (*Tgfb*) and basic fibroblast growth factor (*Fgf2*) knockout and transgenic mice, will be presented to discuss possible reasons for unexpected knockout phenotypes. The conclusions will be that the knockout phenotypes do, in fact, provide accurate information concerning gene function, that we should let the unexpected phenotypes lead us to the specific cell, tissue, organ culture, and whole animal experiments that are relevant to the function of the genes in question, and that the absence of phenotype indicates that we have not discovered where or how to look for a phenotype.

Before entering into how one should interpret unexpected knockout phenotypes and how one should deal with lack of knockout phenotypes, it is necessary to give a brief introduction into how knockout mice are made. For detailed information, the following reviews are suggested (1–4). Transgenic technology has had a long history; thus, an introduction to that technology will not be given here. Rather, the following reviews are suggested (5, 6). At this juncture, it should be noted that, although transgenic vertebrates ranging from fish to bovids have been produced, knockout technology has to date been successful only in mice, even though embryonic stem (ES) cells have been produced from several other species, including hamster (7), rat (8), rabbit (9, 10), pig (11–13), bovine (14, 15), and zebrafish (16). Consequently, the entire discussion will be focused on mice.

Knockout mice are generated by the injection of genetically engineered or gene-targeted ES cells into a mouse blastocyst to generate a chimeric embryo, which in turn can pass on the engineered gene to its offspring. ES cell lines are established from the inner cell mass of a mouse blastocyst, so that when injected into blastocysts, the ES cells can incorporate into the inner cell mass of the recipient blastocysts thereby chimerizing them. Subsequent to transfer of the chimeric blastocysts into uteri of pseudopregnant mice, chimeric mice are born. If the germline of a chimeric mouse is colonized by cells derived from the injected ES cells, the chimera is termed a "germline" chimera. Some of the offspring of the germline chimeras will then carry the engineered gene in their genomes. Gene targeting in ES cells uses the ES cells' DNA repair apparatus to bring about homologous recombination between an exogenous DNA fragment transfected into the ES cell and its homologous region in the genome. Homologous recombination usually results in replacement of the endogenous region with the exogenous fragment, thereby altering the endogenous gene in a prespecified manner. There are many variations on this procedure by which genes can be altered not only to ablate function, but also to make more subtle mutations (17–19). Such procedures can be used to introduce point mutations, remove specific splicing products, switch isoforms, and humanize genes. In addition, technology has recently been

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developed to make conditional and inducible knockouts in which gene function is ablated either in a developmentally specified tissue (20–22) or in an inducible manner (23–26). These techniques, though exciting, will not be further discussed.

**Extensive nonredundancy in the TGFβ family:** Several thousand cell culture studies on the three mammalian transforming growth factor beta proteins (TGFβs 1, 2, and 3) have implicated these growth and differentiation factors in the function of nearly every cell type studied. Expression studies indicated unique and overlapping expression of the three TGFβs (27, 28). For example, overlapping protein localization was found in all gut epithelia, all layers of the skin, all three muscle types, kidney tubules, lung bronchi, cartilage, and bone (Table 1). Together with the fact that all three TGFβs signal through a common TGF type-II receptor (Figure 1), these data strongly suggest considerable redundancy in function. Consequently, it is surprising that, of the >30 phenotypes of the three *Tgfb* knockout mice that we have described (29–31), none appear to be overlapping (Table 2). These results indicate extensive nonredundancy between TGFβ ligands even though there is considerable overlap in expression. Of course, these results do not rule out the possibility of some redundancy in some tissues. Combination of the ligand knockouts would uncover such situations, and it is likely that a few will exist, but 30 nonoverlapping phenotypes for three ligands strongly suggests that a vast number of their functions are not redundant.

There are several possible explanations for how there can be so much overlap in ligand expression and yet so much specific ligand function. First, TGFβs are secreted as latent peptides and must be activated before they can bind receptors (32–35). The mechanism by which this extracellular processing occurs is not well understood and may be different for each TGFβ. Hence, ligand processing presumably determines some functional specificity for the three TGFβs. Second, there is a third type of TGFβ receptor, TGFβR3, that can interact with ligand and receptor types I and II before cytoplasmic signaling can occur, though involvement of TGFβR3 is not essential for signaling (36–38). Association with type III receptors is thought to enhance some TGFβR1 and 2/ligand interactions. Upon ligand binding, the serine/threonine receptor TGFβR2 then associates with and phosphorylates the transmembrane serine/threonine receptor TGFβR1, which in turn initiates a phosphorylation-mediated signaling cascade. Hence, combinatorial receptor/ligand interactions will also determine functional specificity. Third, signaling from TGFβR1 can occur through two cytoplasmic signaling proteins called SMAD2 and 3 (39, 40) and, perhaps, through a third called SMAD5 (41). In addition, SMAD6 and 7 can also interact with the other SMADs to inhibit signaling (42–44). Hence, differential SMAD protein interactions with transcriptional machinery will probably also determine functional specificity for the three TGFβ ligands. Finally, there may be several non-transcriptional signaling pathways for TGFβs. For example, we have found that TGFβ1-deficient platelets from *Tgfb1* knockout mice have impaired platelet aggregation that can be restored by incubating isolated platelets with recombinant TGFβ1 (unpublished observations). Because platelets do not have a nucleus, there must exist a signaling pathway that is nontranscriptional. In summary, given the complexities of ligand processing, receptor interactions, and signaling pathways, it becomes clear why redundancy in TGF1, 2, and 3 function has not been detected at the whole animal level, even though there is considerable overlap in expression of *Tgfb* gene family members. Consequently, if other gene families function with similar complexity, it is likely that, in the final analysis, little functional redundancy will be found within gene families.

Two striking examples of apparent functional redundancy are worth considering. The first involves myogenic genes, and the second involves retinoic acid receptors. Contrary to early interpretations, redundancy does not now appear to be

**Table 1. Protein expression of transforming growth factor beta (TGFβ) 1, 2, and 3**

| Tissue/cell type | TGFβ1 | TGFβ2 | TGFβ3 |
|---|---|---|---|
| Cartilage | | | |
| Perichondrium | +++ | + | ++ |
| Chondrocytes | + | ++ | ++ |
| Bone | | | |
| Periosteum | ++ | - | + |
| Osteocytes | | ++ | ++ ++ |
| Tooth | | | |
| Ameloblasts | + | - | + |
| Odontoblasts | + | ++ | - |
| Pulp | + | +++ | + |
| Muscle | | | |
| Smooth | + | + | ++ |
| Cardiac | + | + | +++ |
| Skeletal | + | ++ | |
| Lung | | | |
| Bronchi | ++ | ++ | ++ |
| Alveoli | - | - | - |
| Blood vessels | | | |
| Endothelium | - | - | ++ |
| Smooth muscle | + | + | +++ |
| Kidney | | | |
| Tubules | ++ | ++ | ++ |
| Basement membrane | - | +++ | - |
| Adrenal | | | |
| Cortex | +++ | +++ | - |
| Medulla | - | - | - |
| Gut | | | |
| Esophageal epithelium | +++ | + | + |
| Gastric epithelium | +++ | + | + |
| Intestinal epithelium | ++ | + | + |
| Basement membrane | - | +++ | - |
| Muscularis | + | + | ++ |
| Liver | | | |
| Capsule | - | - | ++ |
| Parenchyma | - | - | - |
| Megakaryocytes | + | - | ++ |
| Eye | | | |
| Lens epithelium | - | - | - |
| Lens fibers | +++ | + | + |
| Ear | | | |
| Cochlear epithelium | - | + | +++ |
| Basement membrane | - | +++ | - |
| CNS | | | |
| Meninges | + | +++ | + |
| Glia | - | ++ | ++ |
| Choroid plexus | - | - | ++ |
| Skin | | | |
| Periderm | ++ | + | ++ |
| Epidermis | +++ | +++ | +++ |
| Dermis | + | +++ | + |
| Hair follicles | ++ | ++ | + |

The polyclonal antibodies used were specific for residues 4–19 of TGFβ1 and 2 and residues 9–20 of TGFβ3. The avidin-biotin system was used for staining. Data obtained from immunohistochemical study of Pelton et al. (28). Reproduced from *The Journal of Cell Biology*, 1991,115:1091–1105, by copyright permission of The Rockefeller University Press.

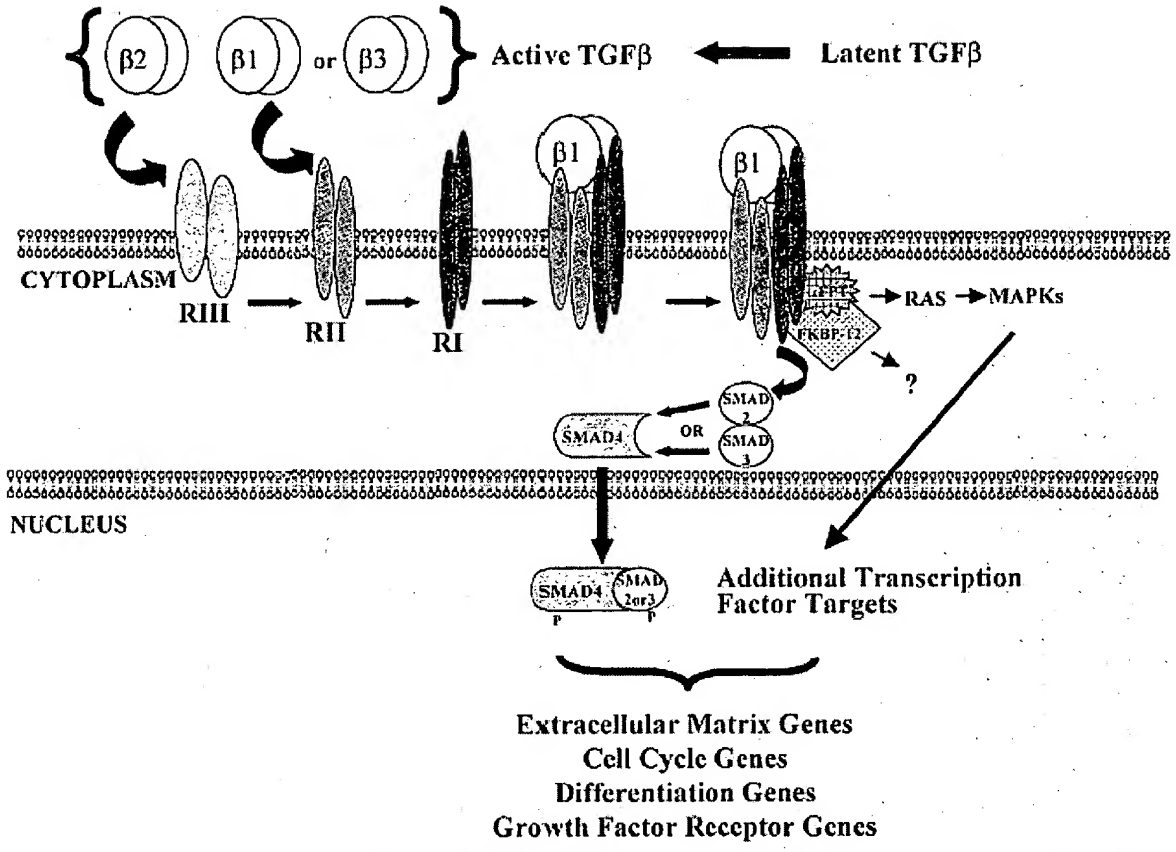


**Figure 1.** TGFβ signaling pathway. The TGFβ ligands, TGFβ1 (β1), TGFβ2 (β2), and TGFβ3 (β3), exist primarily in a latent form in vivo and are activated by mechanisms not yet clear. In general, TGFβ2 interacts with a TGFβ type III receptor (RIII) before interaction with TGFβ type II (RII) and TGFβ type I (RI) receptors; whereas, the TGFβ1 and TGFβ3 ligands can interact directly with the type II receptor. The ligand receptor complexes can then associate with several cytoplasmic molecules, farnesyl protein transferase (FPT) and FK506 binding protein-12 (FKBP-12), being two potential examples. The receptor-ligand complex signals to the nucleus through threonine/serine phosphorylation of a series of SMAD proteins (related to the Drosophila "mothers against decapentaplegic" protein) which then elicit transcriptional regulation of extracellular matrix, cell cycle, differentiation and growth factor receptor genes. The roles of the associated cytoplasmic molecules FPT and FKBP-12 are not clear but are thought to involve RAS pathway signaling and modulation of signaling through the SMAD proteins.

the case for two of the myogenic genes known to be essential for specification of vertebrate skeletal muscle, *Myod* and *Myf5*. Even though the individual knockouts have muscle, and only the combined knockouts do not have muscle (45), it is now clear that each gene functions in the specification of distinct muscle cell lineages. Consequently, in the absence of one source of muscle cells, the other source may compensate for that (46, 47). This should be termed developmental compensation, rather than gene redundancy. On the other hand, with respect to retinoic acid receptors, there is also good evidence for functional redundancy. Similar to the myogenic genes, retinoic acid receptor gene knockout mice have few phenotypes, whereas the combined knockouts have many phenotypes (48, 49). Whether this turns out to be gene redundancy or another case of developmental compensation remains to be determined.

**Lack of phenotype:** As is the case for TGFβ, there also is a multitude of reports indicating that the FGFs 1 and 2 have important roles in numerous cell types and tissues. Consequently, when the *Fgf2* gene was knocked out by gene targeting, it was quite surprising that there was no obvious phenotype (50). The *Fgf2*$^{-/-}$ animals live a long, healthy life, and fertility and fecundity are normal. Even the pituitary gland, which is the best source of FGF2, appears not to have morphologic defects. The only evidence for any developmental abnormalities is found in hematopoiesis (50), where blood platelet counts are high, and in the cerebral cortex (51, 52), where morphometric analysis reveals decreased cell density. Clearly, these abnormalities are minor, compared with expectations. This was all the more evident because our transgenic mice, in which the human *FGF2* gene was ubiquitously overexpressed by the phosphoglycerate kinase pro-

Table 2. Nonoverlapping phenotypes of *Tgfb1*, 2, and 3 knockout mice and the penetrance of those phenotypes

| Knockout mouse phenotype | Penetrance (%)[a] |
|---|---|
| *Tgfb1* | |
| Embryo lethalities | 50 |
| Preimplantation lethality | 50 |
| Yolk sac lethality | 50[b] |
| Adult phenotypes | 50 |
| Multifocal autoimmunity | 100[c] |
| Platelet defect | 100[c,d] |
| Colon cancer | 100[c,d] |
| Failing heart | 100[c,d] |
| *Tgfb2* (all perinatal lethalities) | |
| Heart defects | 100 |
| Ventricular septum defects | 94 |
| Dual outlet right ventricle | 19 |
| Dual inlet left ventricle | 25 |
| Inner ear defect—lacks spiral limbus | 100 |
| Eyes | |
| Ocular hypercellularity | 100 |
| Reduced corneal stroma | 100 |
| Urogenital defects in kidney | |
| Dilated renal pelvis | 30 |
| Agenesis (females only) | 20 |
| Uterine horn ectopia | 40 |
| Testicular ectopia | 100 |
| Testis hypoplasia | 20 |
| Vas deferens dysgenesis | 20 |
| Lung-postnatal | |
| Dilated conducting airways | 100 |
| Collapsed bronchioles | 100 |
| Skeletal defects | |
| Occipital bone | 100 |
| Parietal bone | 100 |
| Squamous bone | 100 |
| Palatine bone (cleft palate) | 22 |
| Alisphenoid bone | 100 |
| Mandibular defects | 100 |
| Short radius and ulna | 100 |
| Missing deltoid tuberosity and third trochanter | 94 |
| Sternum malformations | 25 |
| Rib barreling | 94 |
| Rib fusions | 13 |
| Spina bifida | 100 |
| *Tgfb3* (perinatal lethality) | |
| Cleft palate | 100 |

[a]See Table 3 for background dependency of *Tgfb1* knockout phenotypes.
[b]Described in references 64, 67.
[c]Refers to percentage penetrance among animals that survive to birth.
[d]Unpublished observations.
Details on the remaining phenotypes can be found in the text and in references 29–31, 63.

motor (53), had very short legs, suggesting an important role of FGF2 in bone development, yet the bones of the knockout animals were normal. This apparent discrepancy between the transgenic and knockout mice indicates that some other FGF signals through the same FGF receptor as does FGF2, and that this other FGF is the true ligand that is important in bone development. Another possibility is that there is "developmental compensation" by alternative mechanisms. In other words, the absence of FGF2 may cause developmental abnormalities during bone development that are then compensated for by another developmental pathway. This alternative would not necessarily require a different FGF to be involved.

After we had made our first analysis of the *Fgf2* knockout mouse and did not find an obvious phenotype, it was easy to explain the "lack of phenotype" by invoking redundancy because there are at least 18 known *Fgf* genes. But in hindsight, it now appears more likely that all members of this large gene family have specific functions, even though they signal through receptors encoded by only four receptor genes (54). In *Fgf2* knockout mice, evidence was not found for upregulation of the two ligands most structurally related to FGF2, namely, FGFs 1 and 5 (50). Also, genetic combination of *Fgf2* and *Fgf5* (50) did not reveal redundancy between these similar genes. In addition, further analysis of the mice revealed roles being played in hematopoiesis and vascular tone control (50) as well as in brain development and wound healing (51, 52). Finally, in addition to *Fgf2*, *Fgfs* 3–5, 7, 8 also have been ablated by gene targeting, revealing functions in proliferation of the inner cell mass (*Fgf4*) (55); gastrulation and cardiac, craniofacial, forebrain, midbrain, and cerebellar development (*Fgf8*) (56); brain and inner ear development (*Fgf3*) (57, 58); and two aspects of hair development (*Fgf5* and 7) (59, 60). To date, comparison of *Fgf* knockout phenotypes from 6 of the 18 *Fgf* genes has not turned up overlap except possibly in the cerebellum. Together, these results indicate that each gene has important unique functions. Although a few redundant functions may eventually be found on combination of *Fgf2* with all other *Fgfs* except *Fgf5*, it is clear that 6 of the 18 *Fgf* genes studied by gene targeting have been associated with essentially unique knockout phenotypes.

To summarize, what originally appeared as "lack of phenotype" led many of us to the premature conclusion that other FGFs must have functions redundant to those of FGF2. However, further analysis of *Fgf2* knockout mice has since revealed a wealth of unique functions ranging from thrombocytosis and vascular tone control (50) to brain development and wound healing (51, 52). It is my expectation that further physiologic analysis of the *Fgf2* knockout mouse will reveal functions in the hypertrophic response to hypertension and responses to ischemia/reperfusion injury and bone injury. In the final analysis, it is likely that the major roles of FGF2 may have less to do with getting us to birth than with keeping us alive after birth, whereas several other FGFs clearly have developmental roles.

**Effects of genetic background on phenotypic variation:** From 100 years of mouse genetics, it has become clear that genetic background plays an important role in the susceptibility of mice to many disorders. Therefore, the phenotypes of knockout mouse strains will also have genetic background dependencies, as was first documented by the Magnuson and Wagner groups (61, 62). The *Tgfb1* knockout mice are an exceptional case in point (Table 3). On a mixed (50:50) 129 x CF1 background (CF1 is a partially outbred strain), about half of *Tgfb1* knockout mice die from a preimplantation developmental defect (63), and the other half die of an autoimmune-like multifocal inflammatory disease at about weaning age (29). If the targeted *Tgfb1* allele is backcrossed onto a C57BL/6 background, 99% of all knockout animals die of the preimplantation defect(63). However, if a *Tgfb1* knockout allele is put onto a mixed 129 x NIH/Ola x C57BL/6 background, embryo lethality is observed during yolk sac development, not during preimplantation development (64). With respect to the multifocal inflammatory disorder of *Tgfb1* knockout mice, if the targeted allele is put onto a 129 x CF1 mixed background (50:50), severe inflammation exists only in the stomach (29); on the mixed 129 x

Table 3. Background dependency of *Tgfb1* knockout phenotypes

| Phenotype | Phenotype penetrance on various strains (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 129 x CF1 | 129 x C57 | 129 x C3H | C57 | 129 | C3H | 129xC57x NIH/Ola |
| Preimplantation lethality | 50 | ND | ND | 90 | ND | ND | 0 |
| Yolk sac lethality[a] | 0 | 0 | ND | ND | ND | ND | 50 |
| Autoimmune disease | 50 | 50 | 50 | 1 | ND | ND | 50 |
| Gastric inflammation | 90[b] | 20[b] | ND | ND | ND | ND | ND |
| Intestinal inflammation | 0 | 70[b] | ND | ND | ND | ND | ND |
| Colon cancer[c] | ND | ND | ND | ND | 100 | 0 | ND |

[a]Percentage of knockout animals of a given strain that have the designated phenotype.
[b]For details, see references 64, 67.
[c]Approximately 10% of animals with autoimmune disease have no detectable gastrointestinal tract inflammation.
[d]Unpublished observations.
ND = not determined.

NIH/Ola x C57BL/6 background, the intestines are more severely inflamed than is the stomach (65). Finally, on a predominantly 129 background (129 x CF1; ~97:3), *Tgfb1* knockout mice develop colon cancer if the inflammatory disorder can be eliminated by other genetic manipulations that render the mice immunodeficient (unpublished observations). However, on a predominantly C3H background, immunodeficient *Tgfb1* knockout mice do not develop colon cancer (66). These results suggest that modifier genes exist that can significantly affect the function of TGFβ1 in preimplantation development, yolk sac development, bowel and gastric inflammation, and colon tumor suppression. Progress toward localizing a modifier gene for the yolk sac developmental problem has been made (67).

**What is the best genetic background for knockout mice?** Because background-dependent phenotypic variability will likely be found for most knockout mice, it will be useful to backcross a targeted allele onto several mouse backgrounds to make congenic strains. In this section, it will be argued that putting a targeted allele on a mixed strain background will also provide useful information. This is not to say that congenic strains are not useful. Rather, the point to be made here is that there also are benefits to looking at mixed strain backgrounds. Again, our experience with *Tgfb* knockout mice will be instructive.

*Generating homozygous mutant knockout animals on a mixed genetic background is faster.* The ES cells are nearly always from a 129 strain, and the blastocysts into which the targeted ES cells are injected are nearly always C57BL/6. For reasons unknown, this is a good combination for establishing germline transmission of the injected ES cells. The resulting chimeras can then be crossed with any strain desired, but 129, C57BL/6, or Black Swiss mice are most often used, and CF1 mice were used in the case of our *Tgfb1* knockout mice. Heterozygous offspring from this crossing will then be inbred 129 or F1 hybrids of 129 and one of the other strains. Clearly then, the quickest route to having the knockout allele on an inbred strain is through 129. For the other strains several generations of backcrossing is required, which can take well over a year. Unfortunately, strain-129 mice have low fertility and fecundity. Consequently, the number of offspring per litter is usually fewer than six. Although 129 x C57BL/6 hybrids are more robust, upon backcrossing onto C57BL/6, litter size decreases. To the contrary, the Black Swiss and CF1 strains are robust, and litter size often is in excess of 12. The reason for this is probably because they are not truly inbred strains, but rather are partially outbred through random breeding within their respective strains. Therefore, one of the choices one has is to stay with "pure" genetics at the expense of a lower production rate and considerable delay before generation of experimental animals, or sacrifice some genetic purity to obtain a more efficient production colony. Ideally, one would want to do both, but this often is too expensive.

*Mixed genetic background knockout mice often have a wider range of phenotypes.* The *Tgfb1* knockout mice backcrossed onto either the 129 or C57BL/6 background (congenics) yield only embryo lethality (63, unpublished observations). On the other hand, when the knockout allele is maintained on mixed genetic backgrounds, embryo and adult phenotypes are maintained.

The *Tgfb2* & *Tgfb3* knockout mice provide further examples. The *Tgfb2* knockout mice have more than two dozen congenital defects and die either immediately preceding or during birth, or within 2 h thereafter (30). Table 2 indicates that most of the phenotypes are only partially penetrant. Though it is not documented, it is likely that the penetrance of some of these phenotypes would increase to nearly 100%, and some of the other phenotypes would disappear were we to put the *Tgfb2* knockout allele on inbred backgrounds. Hence, the mixed strain background probably provided more information than would congenic strains.

The *Tgfb3* knockout mice have a cleft palate (31). One colony of *Tgfb3* knockout mice was left as a mixed background (129 x CF1; 50:50) strain, whereas another colony was backcrossed several generations to the C57BL/6 strain. These two colonies had considerable expressivity differences; the inbred colony had more severe clefting than did the mixed background colony. In the latter, expressivity of clefting varied widely from animal to animal. This variable expressivity within the mixed background colony provided us with the opportunity to obtain far more data on development of the cleft palate and was, therefore, more useful for making assumptions about the cellular and molecular mechanisms by which TGFβ3 supports palate fusion. Hence, using the *Tgfb3* knockout mice, the mixed strain background provided more information than did the congenic strain. Consequently, a wider range of penetrance and expressivity of phenotype is a major advantage of investigating knockout phenotypes in mixed background colonies. Further, variable penetrance of phenotype in a mixed background colony suggests that there are modifier genes for each phenotype that could be obtained by linkage studies.

## Conclusions

Questions have been addressed that arose from the last 8 years in which knockout mice have been investigated to analyze gene function at the whole animal level. These questions concern gene redundancy, apparent lack of phenotype in a surprising number of knockout strains, and effects of genetic background on knockout phenotype. Using data obtained principally from *Tgfb* and *Fgf* knockout mice, it is argued that there is probably little redundancy in the genome (i.e., that few genes are dispensable for survival of the species). Apparent lack of phenotype more likely reflects our inability to ask the right questions, or our lack of tools to answer them, than it does a true lack of function. Finally, discussion of genetic background phenotype variability, including variable penetrance and expressivity, was used to present some of the advantages of working with mixed genetic background colonies of knockout mice. For all the examples given here, there are counter examples that must be taken seriously; consequently, these arguments must not be taken as absolutes. For example, if a gene in a particular mouse strain has recently been duplicated, it will most likely be redundant. If one is studying tissue rejection in a knockout mouse, the genetic background obviously must be well defined and preferably inbred. Or, if one wants to use the susceptibility of a particular mouse strain to cancer to investigate the function of the knockout gene in progression of that cancer, the knockout allele must be put on that mouse strain. In general, however, when setting up approaches for investigating a new gene knockout mouse, I believe one would be well advised to assume that: there is little gene redundancy in mammals; there are knockout phenotypes even if none are immediately apparent; and investigating phenotypes in mixed genetic background colonies may not only reveal more phenotypes, but may lead to better understanding of the molecular or cellular mechanism underlying the phenotype, and may lead to modifier gene discovery.

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## References

1. **Mansour, S. L.** 1990. Gene targeting in murine embryonic stem cells: introduction of specific alterations into the mammalian genome. Genet. Anal. Tech. Appl. 7:219–227.
2. **Koller, B. H., and O. Smithies.** 1992. Altering genes in animals by gene targeting. Ann. Rev. Immunol. 10:705–730.
3. **Bradley, A., R. Ramirez-Solis, H. Zheng, *et al.*** 1992. Genetic manipulation of the mouse via gene targeting in embryonic stem cells. Ciba Found. Symp. 165:256–269.
4. **Doetschman, T.** 1994. Gene transfer in embryonic stem cells, p. 115–146. *In* C. A. Pinkert (ed.), Transgenic animal technology: a laboratory handbook. Academic Press, Inc., New York.
5. **Jaenisch, R.** 1988. Transgenic animals. Science 240: 1468–1474.
6. **Hanahan, D.** 1989. Transgenic mice as probes into complex systems. Science 246:1265–1275.
7. **Doetschman, T., P. Williams, and N. Maeda.** 1988. Establishment of hamster blastocyst-derived embryonic stem (ES) cells. Dev. Biol. 127:224–227.
8. **Iannaccone, P. M., G. U. Taborn, R. L. Garton, *et al.*** 1994. Pluripotent embryonic stem cells from the rat are capable of producing chimeras. Dev. Biol. 163:288–292.
9. **Graves, K. H., and R. W. Moreadith.** 1993. Derivation and characterization of putative pluripotential embryonic stem cells from preimplantation rabbit embryos. Mol. Reprod. Dev. 36:424–433.
10. **Schoonjans, L., G. M. Albright, J. L. Li, *et al.*** 1996. Pluripotential rabbit embryonic stem (ES) cells are capable of forming overt coat color chimeras following injection into blastocysts. Mol. Reprod. Dev. 45:439–443.
11. **Wheeler, M. B.** 1994. Development and validation of swine embryonic stem cells: a review. Reprod. Fertil. Dev. 6:563–568.
12. **Shim, H., A. Gutierrez-Adan, L. R. Chen, *et al.*** 1997. Isolation of pluripotent stem cells from cultured porcine primordial germ cells. Biol. Reprod. 57:1089–1095.
13. **Piedrahita, J. A., K. Moore, B. Oetama, *et al.*** 1998. Generation of transgenic porcine chimeras using primordial germ cell-derived colonies. Biol. Reprod. 58:1321–1329.
14. **Cherny, R. A., T. M. Stokes, J. Merei, et al.** 1994. Strategies for the isolation and characterization of bovine embryonic stem cells. Reprod. Fertil. Dev. 6:569–575.
15. **First, N. L., M. M. Sims, S. P. Park, *et al.*** 1994. Systems for production of calves from cultured bovine embryonic cells. Reprod. Fertil. Dev. 6:553–562.
16. **Sun, L., C. S. Bradford, and D. W. Barnes.** 1995. Feeder cell cultures for zebrafish embryonal cells in vitro. Mol. Mar. Biol. Biotechnol. 4:43–50.
17. **Valancius, V., and O. Smithies.** 1991. Testing an "in-out" targeting procedure for making subtle genomic modifications in mouse embryonic stem cells. Mol. Cell. Biol. 11:1402–1408.
18. **Hasty, P., R. Ramirez-Solis, R. Krumlauf, *et al.*** 1991. Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. Nature 350:243–246.
19. **Askew, G. R., T. Doetschman, and J. B. Lingrel.** 1993. Site-directed point mutations in embryonic stem cells: a gene-targeting tag-and-exchange strategy. Mol. Cell. Biol. 13: 4115–4124.
20. **Gu, H., Y. R. Zou, and K. Rajewsky.** 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell 73:1155–1164.
21. **Gu, H., J. D. Marth, P. C. Orban, *et al.*** 1994. Deletion of a DNA polymerase *beta* gene segment in T cells using cell type-specific gene targeting. Science 265:103–106.
22. **Schwenk, F., U. Baron, and K. Rajewsky.** 1995. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res. 23:5080–5081.
23. **Fishman, G. I., M. L. Kaplan, and P. M. Buttrick.** 1994. Tetracycline-regulated cardiac gene expression in vivo. J. Clin. Invest. 93:1864–1868.
24. **Shockett, P., M. Difilippantonio, N. Hellman, *et al.*** 1995. A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. Proc. Natl. Acad. Sci. USA 92:6522–6526.
25. **Kuhn, R., F. Schwenk, M. Aguet, *et al.*** 1995. Inducible gene targeting in mice. Science 269:1427–1429.
26. **No, D., T. P. Yao, and R. M. Evans.** 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc. Natl. Acad. Sci. USA 93:3346–3351.
27. **Pelton, R. W., B. L. Hogan, D. A. Miller, *et al.*** 1990. Differential expression of genes encoding TGFs *beta* 1, *beta* 2, and *beta* 3 during murine palate formation. Dev. Biol. 141: 456–460.
28. **Pelton, R. W., B. Saxena, M. Jones, *et al.*** 1991. Immunohistochemical localization of TGF *beta* 1, TGF *beta* 2, and TGF *beta* 3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. J. Cell Biol. 115:1091–1105.

29. **Shull, M. M., I. Ormsby, A. B. Kier, *et al.*** 1992. Targeted disruption of the mouse transforming growth factor-*beta* 1 gene results in multifocal inflammatory disease. Nature **359:** 693–699.
30. **Sanford, L. P., I. Ormsby, G. A. Gittenberger-de, *et al.*** 1997. TGF *beta* 2 knockout mice have multiple developmental defects that are non-overlapping with other TGF *beta* knockout phenotypes. Development **124:**2659–2670.
31. **Proetzel, G., S. A. Pawlowski, M. V. Wiles, *et al.*** 1995. Transforming growth factor-*beta* 3 is required for secondary palate fusion. Nat. Genet. **11:**409–414.
32. **Flaumenhaft, R., M. Abe, Y. Sato, *et al.*** 1993. Role of the latent TGF-*beta* binding protein in the activation of latent TGF-*beta* by co-cultures of endothelial and smooth muscle cells. J. Cell Biol. **120:**995–1002.
33. **Munger, J. S., J. G. Harpel, F. G. Giancotti, *et al.*** 1998. Interactions between growth factors and integrins: latent forms of transforming growth factor-*beta* are ligands for the integrin *alpha*$_v$*beta*$_1$. Mol. Biol. Cell **9:**2627–2638.
34. **Munger, J. S., J. G. Harpel, P. E. Gleizes, *et al.*** 1997. Latent transforming growth factor-*beta*: structural features and mechanisms of activation. Kidney Int. **51:**1376–1382.
35. **Nunes, I., P. E. Gleizes, C. N. Metz, *et al.*** 1997. Latent transforming growth factor-*beta* binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-*beta*. J. Cell Biol. **136:**1151–1163.
36. **Cheifetz, S., T. Bellon, C. Cales, *et al.*** 1992. Endoglin is a component of the transforming growth factor-*beta* receptor system in human endothelial cells. J. Biol. Chem. **267:** 19027–19030.
37. **Lamarre, J., J. Vasudevan, and S. L. Gonias.** 1994. Plasmin cleaves betaglycan and releases a 60 kDa transforming growth factor-*beta* complex from the cell surface. Biochem. J. **302:**199–205.
38. **Sankar, S., N. Mahooti-Brooks, M. Centrella, *et al.*** 1995. Expression of transforming growth factor type III receptor in vascular endothelial cells increases their responsiveness to transforming growth factor *beta* 2. J. Biol. Chem. **270:**13567–13572.
39. **Heldin, C. H., K. Miyazono, and P. ten Dijke.** 1997. TGF-*beta* signalling from cell membrane to nucleus through SMAD proteins. Nature **390:**465–471.
40. **Kretzschmar, M., and J. Massague.** 1998. SMADs: mediators and regulators of TGF-*beta* signaling. Curr. Opin. Genet. Dev. **8:**103–111.
41. **Bruno, E., S. K. Horrigan, D. Van Den Berg, *et al.*** 1998. The Smad5 gene is involved in the intracellular signaling pathways that mediate the inhibitory effects of transforming growth factor-*beta* on human hematopoiesis. Blood **91:** 1917–1923.
42. **Afrakhte, M., A. Moren, S. Jossan, *et al.*** 1998. Induction of inhibitory Smad6 and Smad7 mRNA by TGF-*beta* family members. Biochem. Biophys. Res. Commun. **249:**505–511.
43. **Nakayama, T., H. Gardner, L. K. Berg, *et al.*** 1998. Smad6 functions as an intracellular antagonist of some TGF-*beta* family members during Xenopus embryogenesis. Genes Cells **3:**387–394.
44. **Itoh, S., M. Landstrom, A. Hermansson, *et al.*** 1998. Transforming growth factor *beta* 1 induces nuclear export of inhibitory Smad7. J. Biol. Chem. **273:**29195–29201.
45. **Rudnicki, M. A., P. N. Schnegelsberg, R. H. Stead, *et al.*** 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell **75:**1351–1359.
46. **Kablar, B., K. Krastel, C. Ying, *et al.*** 1997. MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. Development **124:**4729–4738.
47. **Ordahl, C. P., and B. A. Williams.** 1998. Knowing chops from chuck: roasting myoD redundancy. Bioessays **20:**357–362.
48. **Lohnes, D., M. Mark, C. Mendelsohn, *et al.*** 1994. Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. Development **120:**2723–2748.
49. **Mendelsohn, C., D. Lohnes, D. Decimo, *et al.*** 1994. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development **120:**2749–2771.
50. **Zhou, M., R. L. Sutliff, R. J. Paul, *et al.*** 1998. Fibroblast growth factor 2 control of vascular tone. Nat. Med. **4:**201–207.
51. **Ortega, S., M. Ittmann, S. H. Tsang, *et al.*** 1998. Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. Proc. Natl. Acad. Sci. USA **95:**5672–5677.
52. **Dono, R., G. Texido, R. Dussel, *et al.*** 1998. Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. EMBO J. **17:**4213–4225.
53. **Coffin, J. D., R. Z. Florkiewicz, J. Neumann, *et al.*** 1995. Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. Mol. Biol. Cell **6:**1861–1873.
54. **Ornitz, D. M., J. Xu, J. S. Colvin, *et al.*** 1996. Receptor specificity of the fibroblast growth factor family. J. Biol. Chem. **271:**15292–15297.
55. **Feldman, B., W. Poueymirou, V. E. Papaioannou, *et al.*** 1995. Requirement of FGF-4 for postimplantation mouse development. Science **267:**246–249.
56. **Meyers, E. N., M. Lewandoski, and G. R. Martin.** 1998. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nat. Genet. **18:**136–141.
57. **Mansour, S. L., J. M. Goddard, and M. R. Capecchi.** 1993. Mice homozygous for a targeted disruption of the proto-oncogene int-2 have developmental defects in the tail and inner ear. Development **117:**13–28.
58. **McKay, I. J., J. Lewis, and A. Lumsden.** 1996. The role of FGF-3 in early inner ear development: an analysis in normal and kreisler mutant mice. Dev. Biol **174:**370–378.
59. **Hebert, J. M., T. Rosenquist, J. Gotz, *et al.*** 1994. FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. Cell **78:**1017–1025.
60. **Guo, L., L. Degenstein, and E. Fuchs.** 1996. Keratinocyte growth factor is required for hair development but not for wound healing. Genes Dev. **10:**165–175.
61. **Threadgill, D. W., A. A. Dlugosz, L. A. Hansen, *et al.*** 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science **269:**230–234.
62. **Sibilia, M., and E. F. Wagner.** 1995. Strain-dependent epithelial defects in mice lacking the EGF receptor. Science **269:**234–238.
63. **Kallapur, S., I. Ormsby, and T. Doetschman.** 1999. Strain dependency of TGFβ1 function during embryogenesis. Mol. Reprod. Develop. **52:**341–349.
64. **Dickson, M. C., J. S. Martin, F. M. Cousins, *et al.*** 1995. Defective haematopoiesis and vasculogenesis in transforming growth factor-*beta* 1 knock out mice. Development **121:**1845–1854.
65. **Kulkarni, A. B., C. G. Huh, D. Becker, *et al.*** 1993. Transforming growth factor *beta* 1 null mutation in mice causes excessive inflammatory response and early death. Proc. Natl. Acad. Sci. USA **90:**770–774.
66. **Diebold, R. J., M. J. Eis, M. Yin, *et al.*** 1995. Early-onset multifocal inflammation in the transforming growth factor *beta* 1-null mouse is lymphocyte mediated. Proc. Natl. Acad. Sci. USA **92:**12215–12219.
67. **Bonyadi, M., S. A. Rusholme, F. M. Cousins, *et al.*** 1997. Mapping of a major genetic modifier of embryonic lethality in TGF *beta* 1 knockout mice. Nat. Genet. **15:**207–211.

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